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Enzymatic acidolysis of triolein with palmitic and caprylic acids: Optimization of reaction parameters by response surface methodology

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RESUMEN

Acidólisis enzimática de trioleína con los ácidos palmítico y caprílico: Optimización de los parámetros de la reacción mediante la metodología de superficie de respuesta.

La reacción de acidólisis de la trioleína con los ácidos caprílico y palmítico se realizó utilizando lipasa inmovilizada *Mucor miehei*, específica de sn-1, 3, para producir una grasa de untar baja en calorías compuesta de lípidos estructurados (SL). La metodología de superficie de respuesta se aplica para modelar y optimizar las condiciones de reacción utilizando un factor-cuatro y nivel-cinco de diseño central compuesto. Los factores seleccionados fueron el tiempo (10-24 h), la carga de enzima (10-25% en peso), la relación molar de sustratos (Trioleína:Ácido Caprílico:Ácido Palmítico), (1:1:1-1:2.5:2.5) y la temperatura (45-60 °C). Los SLs producidos se compararon con extractos de grasa de margarina comercial en términos de perfil de fusión y contenido de grasa sólida (SFC). El SL con un pico de fusión a 42 °C y SFC de 40,69% a 0 °C era muy similar a las margarinas suaves. El valor calórico de este SL se determinó teóricamente siendo 37,74 kJ/g. Las condiciones óptimas de reacción encontradas fueron 14 h de tiempo de reacción; una relación molar de sustratos 1:2.1:2.1; una temperatura de 58 °C, y una carga enzima de 15% en peso. En condiciones óptimas el producto contenía 29,68% de AOC, 25,47% de POC, y 3,80% de POP.

PALABRAS CLAVE: Ácido caprílico – Ácido palmítico – Acidólisis enzimática – Metodología de Superficie de Respuesta – *Mucor miehei* – Trioleína.

SUMMARY

Enzymatic acidolysis of triolein with palmitic and caprylic acids: Optimization of reaction parameters by response surface methodology.

An acidolysis reaction of triolein with caprylic and palmitic acids was performed using immobilized sn-1,3 specific lipase from *Mucor miehei* to produce a reduced calorie spreadable structured lipid (SL). Response surface methodology was applied to model and optimize the reaction conditions using a four-factor five-level central composite rotatable design. The selected factors were time (10-24 h), enzyme load (10-25 wt%), substrate mole ratio (Triolein:Caprylic acid:Palmitic acid), (1:1:1-1:2.5:2.5) and temperature (45-60 °C). The produced SLs were compared to fat extracts of commercial margarine in terms of melting profile and solid fat content (SFC). SL with a melting peak of 42 °C and SFC of 40.69% at 0 °C was very similar to soft margarines. The caloric value of this SL was determined as 37.74 kJ/g, theoretically. The optimum reaction

conditions were found as reaction time 14 h; substrate mole ratio 1:2.1:2.1; temperature 58 °C; and enzyme load 15 wt%. Under optimum conditions, the product contained 29.68% COC, 25.47% POC, and 3.80% POP.

KEY-WORDS: Caprylic Acid – Enzymatic acidolysis – *Mucor miehei* – Palmitic Acid – Response Surface Methodology – Triolein.

1. INTRODUCTION

Fats and oils are the major food components and the main energy source of the human body (Adamczek, 2004). Natural oils are mainly triacylglycerols (TAGs) which differ in their physical and nutritional properties and do not always meet all nutritional recommendations or possess the desirable physicochemical properties. Fats and oils are used in human nutrition directly as natural products or, more often, after appropriate modifications (Adamczek, 2004; Gunstone, 2004). Modified lipids that have been restructured in terms of their native composition and/or distribution of fatty acids (FA) in the glycerol backbone are known as structured lipids (SLs) (Foresti and Ferreira, 2010; D'Agostini *et al.*, 2001). SLs combine the unique characteristics of component fatty acids such as melting behavior, digestion, absorption, and metabolism in one triacylglycerol molecule to enhance the role fats and oils play in food, nutrition, therapeutics and health applications (Akoh and Min, 2002; Osborn and Akoh, 2002; Ciftci *et al.*, 2009). Fatty acid composition and their position in the TAG determine the functional and physical properties, metabolic rate, and health benefits of the structured lipid (Koh *et al.*, 2010; Çiftçi *et al.*, 2008; Nieto *et al.*, 1999). The main variation in the fatty acid composition of oils and fats is the chain length and degree of unsaturation of the component FA. This variation in FA composition can dramatically affect the bioavailability and digestibility of oils and fats in infants and adults (Tan and Che Man, 2000). The position of FA in the TAG molecules (sn-1, sn-2, and sn-3) has a significant impact on their metabolism in the body. In general, FAs at the terminal positions of TAG (sn-1 and sn-3) are hydrolyzed by pancreatic lipase and absorbed,

while those at the *sn*-2 position of TAG remain unchanged and are used in the synthesis of a new TAG (Zarevúcka and Wimmer, 2008; Feltes *et al.*, 2009). Therefore, a great deal of attention is focused on the fatty acid present at the *sn*-2 position, which is probably absorbed as 2-monoacylglycerols and serves as a template for reesterification by intestinal cells (Sellappan and Akoh, 2001). The short- or medium-chain fatty acids at the 1- and 3-positions are rapidly oxidized in the liver as readily available energy and are usually not deposited in adipose tissues (Zhou *et al.*, 2001).

The modification of fatty acid composition as well as the regio- and stereo-chemical structure of triacylglycerols improves their nutritive value and changes their physicochemical properties (Adamczek, 2004). The desired melting behavior can be achieved through the interesterification of suitable triacylglycerol mixtures with the use of *sn*-1,3 specific lipases (Schmid and Verger, 1998). When short or medium chain fatty acids and long chain fatty acids are incorporated, they can produce TAGs with good spreadability and temperature stability (Osborn and Akoh, 2002).

The objective of this paper is to synthesize a reduced calorie spreadable SL using triolein. In this respect, an *sn*-1,3 regiospecific lipase catalyzed acidolysis reaction of triolein with caprylic and palmitic acids were carried out. Caprylic acid (CA) and palmitic acid (PA) were targeted to the *sn*-1 and *sn*-3 positions of structured lipids while oleic acid was maintained at the *sn*-2 position for efficient absorption as 2-monoacylglycerols during metabolism. Caprylic acid was added to reduce the caloric value and palmitic acid was used to improve the melting characteristic of the lipid in the acidolysis reaction. This study is the first to combine the unique characteristics of caprylic and palmitic acids and also discuss the melting point, caloric value and compositions of produced TAG in the same system along with the optimization of the reaction conditions with response surface methodology (RSM). Response surface methodology was used to evaluate the effect of several variables in the acidolysis reaction. Response surface methodology is an effective tool for optimizing the process when many factors and interactions affect desired responses (Hunter, 1959). This method has been successfully adapted in many optimization studies (Alim *et al.*, 2008; Wanasundara and Shahidi, 2009).

2. MATERIALS AND METHODS

2.1. Materials

Triolein (OOO, purity $\geq 99\%$), caprylic acid (purity $\geq 99\%$) were obtained from Sigma Chemical Co. (St. Louis, MO). Palmitic acid (purity $\geq 98\%$) was obtained from Merck (Darmstadt, Germany). Immobilized *sn*-1,3 specific lipases (*Rhizopus oryzae*, 367 U/g;

Candida antarctica, 2.1 U/mg; *Mucor miehei*, 140 U/mg; *Pseudomonas fluorescens*, 40 U/g) were purchased from Fluka Chemie GmbH (Steinheim, Germany). Acetone, acetonitrile and *n*-hexane were purchased from Sigma Aldrich. All solvents used were HPLC grade. All other reagents and solvents were of analytical or chromatographic grade.

2.2. Lipase Screening

The acidolysis of triolein with caprylic and palmitic acids was carried out using four kinds of lipases from different sources and analyzed for their ability to incorporate CA and PA into triolein to produce desired TAGs. Immobilized *sn*-1,3 specific lipases (*Rhizopus oryzae*, *Candida antarctica*, *Mucor miehei*, *Pseudomonas fluorescens*) were used as biocatalysts. The reaction conditions of the screening study were chosen as enzyme load, 10 wt%; time, 24 h; reaction temperature, 55 °C and substrate mole ratios (Triolein:CA:PA), 1:1:1. *sn*-1,3 specific lipase from *Mucor miehei* producing desired TAGs at higher amounts was selected for further studies.

2.3. Effect of Caprylic Acid: Palmitic Acid Mole Ratio on the Acidolysis Reaction

A preliminary study was implemented prior to the RSM work to understand the effect of different CA:PA mole ratios on the lipase-catalyzed acidolysis reaction. The acidolysis reactions were performed as enzyme load, 10 wt %; time, 24 h and temperature, 55 °C with variable substrate mole ratios (Triolein:CA:PA). The amount of triolein was fixed at 0.1 mmol in each reaction. The molar ratio of CA:PA was changed from 1:1 to 1:4 and from 1:1 to 4:1. The samples were analyzed using reversed phase HPLC. The percentage of TAG obtained was calculated and an optimum CA:PA ratio was chosen on the basis of a higher incorporation of fatty acids to produce the desired TAG content. The molar ratio of CA:PA was chosen as 1:1 to study in reaction systems. The selected CA:PA ratio was fixed and their ratio to triolein (Triolein:CA:PA) ranged between 1:1:1 and 1:3:3 at RSM design.

2.4. Synthesis of Structured Lipid

The acidolysis of triolein was performed in 100-mL tightly closed-screw capped flasks. Triolein (0.1 mmol) and the corresponding ratio of caprylic and palmitic acids were mixed in 2 mL of *n*-hexane. The specified amount of enzyme (wt %) was added as given in Table 1. The values of time and temperature were set according to an experimental central composite design (Table 1). The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm. At the end of the reaction, the product was separated from the enzyme by decanting. The *n*-hexane was evaporated in a water bath at 60 °C and the mixtures were

Table 1
A five-level, four factorial central composite rotatable design (CCRD) and TAG% composition of the SLs obtained by acidolysis of triolein with caprylic and palmitic acids under the conditions generated by RSM

Run	Enzyme load (wt %)	Reaction temperature (°C)	Time (h)	Substrate Mole Ratio ¹	COC %	COO %	POC %	OOO %	POO %	POP %	Energy (kJ/g)
1	10.0	45.0	10	1.00	1.70	25.15	1.78	46.43	24.13	0.81	39.17
2	25.0	45.0	10	1.00	2.43	24.50	5.45	40.05	24.79	2.78	39.07
3	10.0	60.0	10	1.00	4.85	24.69	9.03	32.23	25.80	3.40	38.91
4	25.0	60.0	10	1.00	5.87	23.98	10.06	30.58	24.61	4.90	38.86
5	10.0	45.0	24	1.00	2.24	22.62	4.26	46.19	23.49	1.20	39.14
6	25.0	45.0	24	1.00	3.05	22.65	4.47	43.97	22.99	2.87	39.10
7	10.0	60.0	24	1.00	4.73	21.86	10.06	31.61	26.36	5.38	38.92
8	25.0	60.0	24	1.00	6.65	24.20	14.57	24.75	23.25	6.58	38.74
9	10.0	45.0	10	2.50	14.50	27.27	14.03	29.42	12.08	2.70	38.48
10	25.0	45.0	10	2.50	23.10	28.25	18.80	10.33	14.83	4.69	38.07
11	10.0	60.0	10	2.50	19.89	28.91	16.56	20.08	11.96	2.60	38.23
12	25.0	60.0	10	2.50	29.10	27.55	25.15	2.61	10.47	4.38	37.74
13	10.0	45.0	24	2.50	22.11	32.73	10.64	18.80	12.37	3.35	38.22
14	25.0	45.0	24	2.50	29.10	31.09	18.42	4.42	12.17	4.80	37.84
15	10.0	60.0	24	2.50	28.23	29.65	20.11	6.70	11.03	4.28	37.86
16	25.0	60.0	24	2.50	33.83	25.88	25.27	1.85	6.79	6.38	37.62
17	2.5	52.5	17	1.75	2.03	28.66	5.01	49.23	13.18	1.89	39.06
18	32.5	52.5	17	1.75	25.02	28.19	20.13	10.76	10.7	5.20	37.99
19	17.5	37.5	17	1.75	23.31	35.20	12.01	15.75	11.93	1.80	38.12
20	17.5	67.5	17	1.75	23.44	36.30	17.73	6.01	11.76	4.76	37.98
21	17.5	52.5	3	1.75	12.10	35.41	13.19	14.70	21.94	2.66	38.45
22	17.5	52.5	31	1.75	16.60	34.27	13.14	9.36	22.08	4.55	38.31
23	17.5	52.5	17	0.25	1.02	4.93	1.85	62.11	28.11	1.98	39.45
24	17.5	52.5	17	3.25	30.78	14.91	28.17	13.27	7.67	5.20	37.82
25	17.5	52.5	17	1.75	28.30	29.51	20.10	6.1	12.68	3.31	37.87
26	17.5	52.5	17	1.75	33.20	28.72	24.89	0.2	8.94	4.45	37.78
27	17.5	52.5	17	1.75	25.60	30.86	24.70	4.57	10.96	3.31	37.85
28	17.5	52.5	17	1.75	26.70	29.58	26.00	0.72	12.57	4.45	37.81
29	17.5	52.5	17	1.75	27.20	30.17	25.30	2.48	11.75	3.10	37.79
30	17.5	52.5	17	1.75	29.80	30.85	24.50	0.9	10.56	3.39	37.71

¹ Substrate mole ratio refers to Triolein:Caprylic acid:Palmitic acids, ranging between 1:0.25:0.25 and 1:3.25:3.25. C caprylic acid; O oleic acid; P palmitic acid.

stored at -20°C for subsequent analysis. All reactions were performed in duplicate and the average values were reported.

2.5. Removal of Free Fatty Acids

The reaction products were neutralized to remove free fatty acids in a similar manner as in de Araújo *et al.* (2011). The reaction mixture (0.5 mg) was mixed with 20 ml *n*-hexane and 0.5 mL phenolphthalein. Then 0.5 N KOH in 20% (v) ethanol was added until a pink color was observed. The upper layer was decanted. The lower layer was washed with *n*-hexane and the upper layer was

again decanted. The remaining *n*-hexane was evaporated and a fatty acid free reaction product was obtained.

2.6. Separation of the Fat Phase of Margarine

10-15 g of margarine were incubated at 55°C for 40 min in a separatory funnel. The upper phase was separated and used in DSC analysis.

2.7. Analysis of TAG Content

The TAG composition of the reaction product was determined by SCL-10A HPLC system

(Shimadzu, Japan) using the method proposed by the AOCS Official Method Ce 5b-89. The analyses were carried out isocratically with a mobile phase consisting of 64:36 (v/v) acetone/acetonitrile. Oil was diluted in acetone, filtered and injected into the column (Sphereclone 5 μ ODS (2), 250 \times 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 \times 3-mm id) of the same phase and eluted at a flow rate of 1.0 mL min⁻¹. The column temperature was set at 50°C and elution was monitored with an RID-10A (Shimadzu, Japan). The total analysis time was 30 min. All triacylglycerol contents were expressed as weight percent of the total weight of the sample. All analyses were performed in duplicate and average values were reported.

2.8. Thermal Analysis by DSC

The melting profile and SFC of triolein, margarine fats and structured lipids were analyzed by DSC (Perkin Elmer DSC-6, Norwalk, CN, USA). The DSC instrument was calibrated with indium (m.p. 156.6°C, $\Delta H_f = 28.45$ J/g). Nitrogen was used as the purge gas and flowed at 40 mL min⁻¹. A sample was completely melted at 80°C before being weighed (5-10 mg) into an aluminium pan which was then sealed. An empty, hermetically sealed aluminum pan was used as reference. The previous thermal history of the sample was erased by heating the sample to 80°C in the DSC instrument and holding it for 10 min. The sample was then cooled to -60°C at a rate of 5°C min⁻¹ and held at -60°C for 10 min. At the end of the cooling, the sample was heated at 5°C min⁻¹ to 80°C. The onset (T_{onset}) and peak temperatures (T_{peak}) were determined according to the melting profile. The SFC was calculated at various temperatures from the DSC heating thermogram data by partial integration according to Nassu and Goncalves (1995). All DSC values reported are the average of two scans.

2.9. Experimental Design

A five-level, four factorial central composite rotatable design (CCRD) was employed to study the responses, weight percent of TAG yields (COC, COO, POC, OOO, POO, and POP), effects of reaction factors on the production of TAGs and to optimize the reaction conditions to obtain the desired TAG mixture. CCRD was composed of 30 experiments consisting of 16 axial points, 8 star points, and 6 center points (Table 1). The star points provide the estimation of curvature in the models. Six replicate runs at the center point of the design were performed to allow for the estimation of pure error.

The independent variables were selected as reaction time (Ti), enzyme load (En), substrate molar ratio (Sr) and reaction temperature (Te). The levels of the independent variables were defined as Ti, 10-24 h; En, 10-25 wt%; Te, 45-60°C and Sr,

(Triolein:CA:PA), 1:1:1-1:2.5:2.5. Although the substrate mole ratio (Triolein:CA:PO) in the reaction mixtures varied from 1:1:1 to 1:2.5:2.5, the molar ratio between fatty acids (CA:PA) was fixed at 1:1.

2.10. Data Analysis and Optimization by Response Surface Methodology

The data obtained were analyzed using RSM (Stat-Ease, Design-Expert software, version 7). ANOVA, regression analysis and model generation were used to evaluate the effects of factors and to optimize reaction conditions. The level of significance for all tests was set at 95 % confidence level. The goodness of the models established was determined using the coefficient of determination, R^2 , together with the absolute average deviation values and ANOVA (Arifin *et al.*, 2010).

The first- or second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the equation (1):

$$Y_i = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^4 \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y_i ($i = 1 - 6$) are the responses for the weight percent of the produced TAGs, namely, COC (Y_1), COO (Y_2), POC (Y_3), OOO (Y_4), POO (Y_5), POP (Y_6). β_0 , intercept, β_i , first-order model coefficients, β_{ii} , quadratic coefficients for the i^{th} variable, β_{ij} , interaction coefficients for the interaction of variable i and j , and X_i are independent variables and ε is the random error.

2.11. Calorie Value Determinations of Structured Lipids

The TAG compositions of the structured lipids obtained from each run are shown in Table 1. The heat energy values of fatty acids were estimated using the equation (2) proposed by Taguchi *et al.* (2001).

$$-\Delta H_c (\text{fatty acid}) = 0.653n - 0.166d - 0.421 \quad (2)$$

where $-\Delta H_c$ is the heat of combustion in MJ/mol, n is the number of carbon atoms/molecule of the fatty acid, and d is the number of double bonds/FA.

The heat of combustion of each produced TAG was also determined separately in MJ/ grams of fatty acid in 100 g TAG using the approach of Livesey (1984), according to the equation (3):

$$-\Delta H_c (\text{triacylglycerol}) 1.66 = \sum_{FA_i}^{FA} \frac{B'}{BD} + \sum_{FA_i}^{FA} \frac{B'}{D} = \frac{B'}{D} \quad (3)$$

in which B' is g fatty acid /100 g TAG total fatty acid, D is the molecular weight of the corresponding fatty acid and I is $-\Delta H_c$ of the corresponding fatty acid. The constant 1.66 is the heat of combustion (MJ/mol) for glycerol. $-\Delta H_c (\text{triacylglycerol})$ is the potential metabolizable energy of the TAG. To give

$-\Delta H_c(\text{triacylglycerol})$ in MJ/g TAG, $-\Delta H_c(\text{triacylglycerol})$ according to equation (3) was divided by the molecular weight of the triacylglyceride/grams of fatty acids in 100 g TAG.

Caloric values of SLs produced from each run were calculated according to their TAG composition using equation (4):

$$-\Delta H_{c,SL} = X_{coc} * \Delta H_{c,coc} + X_{coo} * \Delta H_{c,coo} + X_{poc} * \Delta H_{c,poc} + X_{ooo} * \Delta H_{c,ooo} + X_{poo} * \Delta H_{c,poo} + X_{pop} * \Delta H_{c,pop} \quad (4)$$

here, $-\Delta H_{c,SL}$ is the caloric value of the produced structured lipids in kJ/g structured lipid, X represents the fraction of TAG/g structured lipid, and ΔH_c is the heat of combustion of TAG in kJ/g TAG and $-\Delta H_{c,SL}$ is the energy value of the produced structured lipids in kJ/g structured lipid.

3. RESULTS AND DISCUSSION

3.1. Lipase Screening

In the acidolysis of triolein with caprylic and palmitic acids using *sn*-1,3 specific lipase there are six possibilities of TAG components, namely, COC, COO, POC, POO, POP and OOO. COC, POC and POP are the most desired triacylglycerols to reduce the caloric value of SL with a spreadable characteristic. COC is the most effective TAG in reducing calories, POP is the most effective one in increasing melting point and POC combines the unique characteristics of caprylic and palmitic acids in one TAG.

Four enzymes from different sources were analyzed for their ability to produce the desired triacylglycerols under the same acidolysis conditions. Figure 1 shows the effect of different lipases on percent yield of TAGs. The lipase from *P. fluorescence* showed the lowest catalytic activity and gave lowest yields of the desired structured TAGs. The *mucor miehei* catalyzed acidolysis reaction promised the highest incorporation of caprylic and palmitic acids into triolein at *sn*-1, 3 positions and resulted in the lowest percent residual triolein. The residual triolein was lower for *M. miehei* lipase than *C. antarctica* although the percent of desired TAGs is slightly higher for *C. antarctica*. The positional specificity of *C. antarctica* depends on the reactants. In some reactions, *C. antarctica* shows a 1,3-positional specificity, whereas in other reactions, the lipase functions as a non-positional-specific lipase (Seriburi and Akoh, 1998a). In our study, lipase from *C. antarctica* behaved as an *sn*-1,3 specific lipase enzyme. Due to the percent yield of structured triacylglycerols and economic considerations, *M. miehei* was selected as the catalyst for further acidolysis reactions among the analyzed lipases. Indeed, the lipase from *Rhizomucor miehei*, formerly *Mucor miehei*, might be considered the most active catalyst in the acidolysis reactions of triolein and a mixture of equimole amounts of n-3 FA (Hamam and Shahidi, 2007).

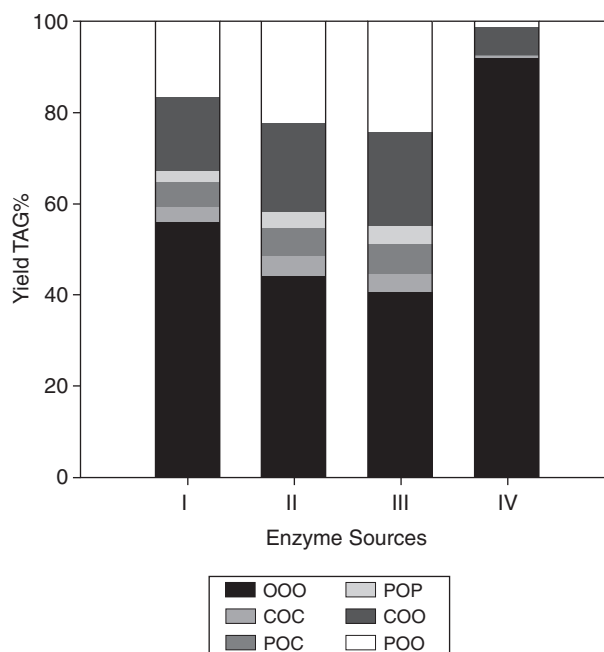


Figure 1

Effect of different lipases on the production of structured TAGs and residual triolein. (I) *R. oryzae*, (II) *C. antarctica*, (III) *M. miehei*, (IV) *P. fluorescence*; POO 1-palmitoyl-2,3-dioleoyl-glycerol, COO 1-capryloyl-2,3-dioleoyl-glycerol, POP 1,3-dipalmitoyl-2-oleoyl-glycerol, POC 1-palmitoyl-2-oleoyl-3-capryloyl, COC 1,3-capryloyl- 2- oleoyl-glycerol, OOO triolein.

3.2. Diagnostic Checking of Fitted Models

RSM was implemented to model the six responses, namely, COC, COO, POC, POO, and POP and residual OOO wt%. The data of responses obtained from the reactions under different conditions as defined in CCRD are shown in Table 1. The models predicted for COC, COO, POC, OOO, POO, and POP wt% were significant at the 99% confidence level and the lack of fits were not significant ($P < 0.05$) with high coefficients of determinations (R^2) between 0.92 and 0.96. The high values for the coefficient of determination, significance of model ($P < 0.001$) and non-significant lack of fit ($P < 0.05$) indicate that the models were a good fit. The best fitting quadratic models were determined by regression and backward elimination by means of the elimination of insignificant factors and interactions in the models. Coefficients and P values for all responses are presented in Table 2.

3.3. Effect of Parameters and Response Surface Plotting

The coefficients obtained for the responses showed that enzyme load, temperature, and substrate mole ratio were significant ($P < 0.001$) and affected the production of the desired TAGs (COC, POC and POP) positively but these parameters were more effective on POC and POP (Table 2). This can be related with the substrate

Table 2
Regression coefficients of predicted quadratic polynomial models for response variables

	Coefficients					
	COC	COO	POC	OOO	POO	POP
Intercept	28.47	29.9	24.2	2.49	12.2	3.7
Linear						
En	3.40 ^a	-0.24 ^{ns}	2.75 ^a	-6.24 ^a	-0.51 ^{ns}	0.84 ^a
Te	1.50 ^c	-0.22 ^{ns}	2.68 ^a	-4.53 ^a	-0.29 ^{ns}	0.86 ^a
Ti	1.53 ^c	-0.07 ^{ns}	0.29 ^{ns}	-1.84 ^{ns}	-0.41 ^{ns}	0.51 ^a
Sr	9.53 ^a	2.57 ^a	5.91 ^a	-12.4 ^a	-6.03 ^a	0.49 ^a
Interaction						
En*Te	-	-	-	-	-0.80 ^{ns}	-
En*Ti	-	-	-	-	-	-
En*Sr	1.67 ^{ns}	-	1.06 ^{ns}	-2.42 ^c	-	-
Te*Ti	-	-	-	-	-	0.38 ^b
Te*Sr	-	-	-	-	-	-0.66 ^a
Ti*Sr	1.51 ^{ns}	0.90 ^a	-	-	0.99 ^c	-
Quadratic						
En²	-4.12 ^a	-0.53 ^{ns}	-3.06 ^a	7.16 ^a	-	-
Te²	-1.65 ^c	1.30 ^a	-2.49 ^a	2.38 ^c	-	-
Ti²	-3.91 ^a	1.07 ^a	-2.91 ^a	2.67 ^b	2.90 ^a	-
Sr²	-3.52 ^a	-5.16 ^a	-2.45 ^a	9.09 ^a	1.87 ^a	-
R²	0.94	0.96	0.95	0.95	0.94	0.92

En enzyme load; Te temperature; Ti time; Sr substrate mole ratio; ^{ns} not significant even at 5% level; ^a Significant at 0.001; ^b Significant at 0.01; ^c Significant at 0.05.

specificity of lipase depending on incubation temperature. Palmitic acid may be a poor substrate at lower temperatures and become better at higher temperatures. The trends of response surface plots were in line with the results of the model coefficient. In Figure 2a-b, it is seen that increasing substrate mole ratio at constant enzyme load caused an increase in the yield of COC and POC, and after a critical point the yield of COC and POC remained constant. This critical point probably represents the enzyme saturated with acid. Similar trends were observed for enzyme load in the yields of COC and POC while the other parameters remained constant (Figure 2a-b). After enzyme load reached its optimum at 20%, a very slight decrease was observed. In this study, it was found that substrate mole ratio was the most significant factor for COC and POC while temperature was the most significant for POP. Zhou *et al.* (2001) and Lumor and Akoh (2005) also found that substrate mole ratio was the most important factor affecting the incorporation of fatty acid into oil. Enzyme load and temperature affect the yield of COO and POO negatively, although not significantly.

Temperature was more effective on the yields of POC and POP than that of COC (Table 2). This

statistical result reflected the response surface plots (Figure 2d-f). While a distinct increase was observed for the percentage of POC and POP with increasing temperature at constant parameters, a very small increase was observed for COC in the plots. The optimum temperatures for the maximum yields of desired TAGs were 56 °C for COC and POC and 60 °C for POP. The optimum temperature recommended by the manufacturer for maximum *Rhizomucor miehei* lipase activity is between 30 and 70 °C (Cheong *et al.*, 2007). Similarly, Sellappan and Akoh (2001) found that the optimum temperature for the incorporation of caprylic acid was 55 °C and also explained that the sustained incorporation of stearic acid at all temperatures was caused by the higher selectivity of IM60 toward stearic acid. In our study, as a long chain fatty acid, palmitic acid also showed the same trend as stearic acid with *sn*-1,3 specific lipase from *Mucor miehei*. High temperatures would be more effective for the incorporation of palmitic acid due to its high melting point (63 °C). Response surface plots for POP showed a two dimensional surface structure (Figure 2c,f). The highest yields for POP were observed at the highest values of parameters in the studied range.

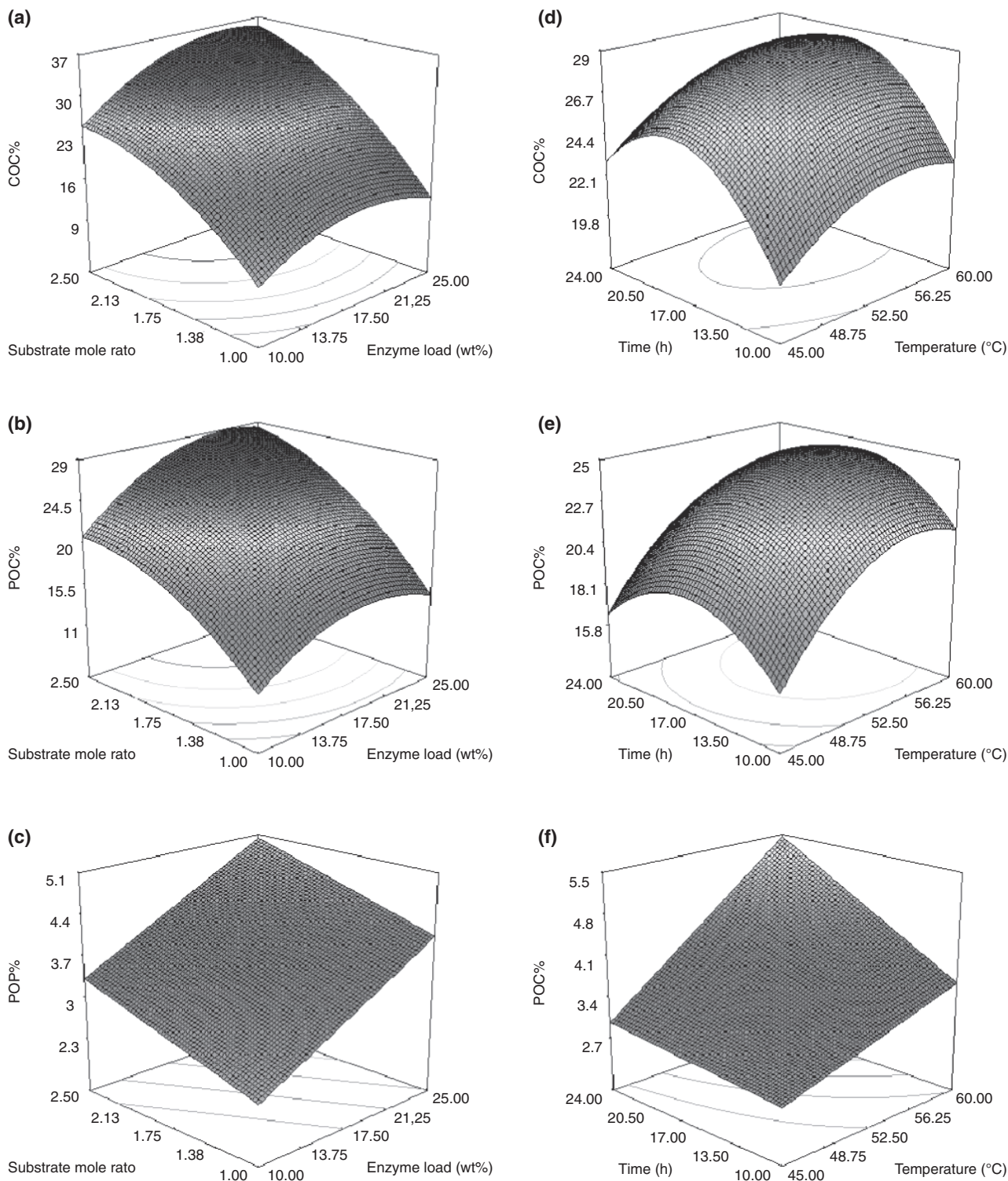


Figure 2

Response surface plots for COC, POC and POP%. (a), (b), (c) substrate mole ratio versus enzyme load (wt%); Time: 17 h and temperature: 52.5 °C; (d), (e), (f) time versus temperature; Enzyme load: 17.5% and substrate mole ratio: 1.75.

Time was also effective in the production of the desired TAGs. Increasing the time caused an increase in the yields of COC, POC and POP but this increase was not statistically significant for POC. However, in Figure 2d-e, it is seen that the yields reached their optimum at 17 h and thereafter there was a slight decrease in the yield of COC and POC. This result is in agreement with the

study of Arifin *et al.* (2010) in which it was reported that the esterification reaction of glycerol with capric and stearic acids reached the equilibrium point at 16 h of time. Time affects COO and POO negatively, but this affect is not significantly important.

Increasing the values of parameters in the studied range caused a decrease in the percentage

of residual OOO as expected. This decrease in OOO was significant for all parameters ($P < 0.001$) except for time. Substrate mole ratio was the predominant parameter and enzyme load, temperature and time followed substrate mole ratio in the decreasing effectiveness order. Hence, it could be said that an increase in enzyme load, time, temperature, and substrate mole ratio gave rise to the percent composition of desired TAGs and reduced the amount of COO and POO.

It is seen that some linear, interaction or quadratic terms were not eliminated by backward elimination to maintain the hierarchy of the model although they were statistically nonsignificant. The presence of non significant interaction terms in Table 2 were the reason for this elimination principle. Most of the interaction terms were found nonsignificant in the response models. Koh *et al.* (2010) reported that most of the interactions between these variables were found to be insignificant at 99% confidence level. The En*Sr interaction was only significant for the residual yield of OOO and caused a decrease in the amount of triolein. The interaction terms of Te*Ti and Te*Sr for the percentage of POP were highly significant ($P < 0.001$). Another interaction term of Ti*Sr was eliminated in the models for the percentages of COC, POC, OOO and POP by the backward elimination quadratic model while it affected COO and POO significantly. Quadratic terms of the parameters were found significant for the COC and POC. However, the quadratic terms of parameter were eliminated since they were statistically nonsignificant for POP%.

3.4. Melting Point and Solid Fat Content

Some structured lipids were selected from the experimental design based on their TAG composition and these SLs were synthesized in gram scale. These structured lipids were analyzed for melting profile and solid fat content and compared with commercially available margarine fat extracts. Only the results of SL obtained from run 12 which showed the most similarities to the properties of margarine fat were given in Tables 3 and 4.

The onset and melting peak temperatures for margarine fats and structured lipids are shown in Table 3. The melting point of pure triolein was found as -12°C and 6.72°C with two endothermic peaks in agreement with Ilyasoglu and Ozcelik (2011) who observed the melting point of triolein at $\sim 5^{\circ}\text{C}$. Hagemann and Tallent (1972) observed that triolein melted at -12°C and -5°C and Seriburi and Akoh (1998b) reported that triolein melted at -15.2 and -2.4°C with two peaks. SL also gave two peaks with peak temperatures at -13.5 and 42.09°C , respectively. The second peak region probably defined the palmitic acid incorporated TAG fraction while the first peak region depicted the caprylic acid incorporated TAG and triolein fraction. The onset

Table 3
Melting profile of structured lipid, triolein and fat extracts of commercial margarines

Sample	Peak	Tonset ($^{\circ}\text{C}$)	Tpeak ($^{\circ}\text{C}$)
BU	I	15.30	20.31
	II	31.23	36.99
A	I	-26.73	-19.30
	II	16.21	39.39
B	I	-20.47	-13.89
	II	13.66	36.47
C	I	-25.99	-20.13
	II	13.59	33.49
D	I	-23.94	-15.44
	II	17.59	39.54
E	I	-25.88	-19.25
	II	12.95	34.62
F	I	-18.48	-11.59
	II	6.95	24.12
SL	I	-27.73	-13.50
	II	28.95	42.09
Triolein	I	-18.23	-12.00
	II	0.87	6.72

BU fat extract of butter; A, B, C, D fat extracts of soft margarines; E, F fat extracts of hard margarines; SL structured lipid obtained from run12 of experimental design.

temperature of the first peak for SL (-27.73°C) was very similar to that of margarine fat extracts A (-26.73°C), C (-25.99°C), and D (-23.99°C) and its melting peak temperature (42.09°C) was very close to fat extracts A (39.39°C) and D (39.54°C) (Table 3).

Table 4 shows the solid fat content profiles for margarine fats, pure triolein and SL. Changes in the TAG composition of the triolein after acidolysis resulted in changes in SFC. The SFC of triolein was higher than SL at lower temperatures (-20 - 4°C) but lower at higher temperatures (10 - 35°C). Incorporated palmitic acids were the possible reason for the improved solid fat content. Our results indicate that the SFC of SL showed a similar trend with soft margarine fats, especially D, at various temperatures. The SFC of SL at 35°C was higher than all margarines except E. This means the produced structured lipid is melted more slowly than the other margarines. SL had 40.69% SFC at 0°C , 30.63% at 10°C , and 27.73% SFC at 20°C , which designates a suitable SFC profile for good plasticity. Lida and Ali (1998) reported that an SFC not greater than 32% at 10°C is essential for good spreadability at refrigeration temperature and an SFC not less than 10% at 20°C is essential for product stability and resistance to oil exudation at room temperature.

Table 4
Solid fat content values of structured lipid, triolein and fat extracts of commercial margarines

Samples	SFC (%) at °C								
	-20	-10	0	4	10	20	25	30	35
BU	100	99.82	95.47	91.64	82.65	54.95	34.17	20.74	9.85
A	78.48	48.53	32.27	29.02	25.82	22.34	17.96	13.33	8.52
B	94.94	55.93	31.20	19.18	24.88	19.32	14.73	10.11	6.27
C	81.86	41.14	27.86	26.17	24.69	20.34	16.12	11.42	6.27
D	91.41	64.75	44.61	39.53	33.88	26.50	21.11	15.73	11.23
E	100	94.83	82.53	78.78	69.13	53.31	41.26	28.20	18.75
F	100	95.72	89.71	88.58	77.01	52.68	34.47	18.75	9.09
SL	87.66	63.49	40.69	35.71	30.63	27.73	25.87	22.35	16.99
Triolein	100	94.98	77.29	94.08	21.93	0.12	0	0	0

SFC solid fat content; BU fat extract of butter; A, B, C, D fat extracts of soft margarines; E, F fat extracts of hard margarines; SL structured lipid obtained from run12 of experimental design.

3.5. Caloric Values of Structured Lipids

The theoretical caloric values of the produced TAGs after acidolysis were calculated from the amount of fatty acids and glycerol in one mol TAG. The energy values of TAGs were determined as COC (36.28 kJg⁻¹), COO (38.30 kJg⁻¹), POC (37.69 kJg⁻¹), OOO (39.65 kJg⁻¹), POO (39.46 kJg⁻¹) and POP (39.21 kJg⁻¹). Then the caloric values of the structured lipids from each run were calculated based on their TAG composition. The results obtained for structured lipids are given in Table 1. Taguchi *et al.* (2001) reported that the calculated energy values were in agreement with the energy values measured by the bomb calorimeter in their study. In our study, the acidolysis reaction was deduced with a tolerable decrease in the caloric value of triolein. The SL from run 12 resulted in a decrease from 39.65 kJg⁻¹ to 37.74 kJg⁻¹ which represents approximately a 5% decrease in the caloric value compared to triolein.

3.6. Optimization

The optimal conditions for the lipase catalyzed acidolysis reaction of triolein, caprylic and palmitic acids were predicted using Design Expert Software 7.0. Optimization was based on melting point, solid fat content and caloric value requirements to produce a reduced calorie spreadable structured lipid from triolein. The SFC and melting point of margarine fat extracts served as target criteria to make the properties of structured lipid similar to those of margarine fat extracts. The target response intervals were defined considering the TAG compositions which satisfied the desired properties. The necessary response intervals were set as COC (25-30%); POC (25-30%) and POP (3-4%). Our observations showed that the TAG composition did not change after 15 wt% enzyme load. Thus, En was kept at 15% due to the cost considerations of the industry. The recommended optimal conditions

were enzyme load 15 wt%, reaction temperature 58 °C, substrate mole ratio (1:2.1:2.1), and time 15 h.

4. CONCLUSION

This study showed that immobilized *sn*-1,3 specific lipase from *Mucor miehei* was the most effective catalyst among the lipases examined to obtain SL. The caloric value of SL with melting characteristics similar to that of commercial soft margarine fat extracts was reduced by approximately 5% compared to triolein. Optimization of the acidolysis reaction of triolein with two fatty acids was successfully performed by response surface methodology for the production of a structured lipid with the desired properties. A structured lipid with the desired properties can be obtained at 15 wt% enzyme load, reaction temperature of 58 °C, reaction time at 15 h, and substrate mole ratio (Triolein:CA:PA) of 1:2.1:2.1 with a TAG composition of COC (29.68%), POC (25.47%) and POP (3.80%). The results of this model system illustrate that it is possible to produce a reduced calorie spreadable fat from vegetable oils rich in triolein.

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